Diurnal expression of hetR and diazocyte development in the filamentous non-heterocystous cyanobacterium Trichodesmium erythraeum

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The marine non-heterocystous cyanobacterium Trichodesmium fixes atmospheric N₂ aerobically in light. In situ immunolocalization/light microscopy of NifH revealed that lighter, non-granulated cell regions observed correspond to the nitrogenase-containing diazocyte clusters in Trichodesmium IMS101. The number of diazocyte clusters per trichome varied from 0 to 4 depending on trichome length. The constant percentage of diazocytes (approx. 15%) in cultured strains and five natural populations suggests a developmentally regulated differentiation process. Real-time RT-PCR showed that ntcA, encoding the global nitrogen regulator in cyanobacteria, and hetR, the key regulatory gene in heterocyst differentiation, are both constitutively expressed during a 12 h/12 h light/dark cycle. hetR in addition showed a distinct peak in the dark (close to midnight) while nifH expression commenced 6–8 h later. The expression of all three genes was negatively affected by addition of ammonia. Some early heterocyst differentiation genes were also identified in the genome of Trichodesmium. The data suggest that hetR and ntcA may be required for development and function of diazocytes in Trichodesmium.

INTRODUCTION

Trichodesmium spp. are marine filamentous cyanobacteria that lack the ability to form akinetes, heterocysts and hormogonia. Therefore, they are classified as undifferentiated filamentous cyanobacteria (Rippka et al., 1979), but this has been questioned after the discovery of the nitrogenase-containing diazocytes (Bergman & Carpenter, 1991; Fredriksson & Bergman, 1997). Members of the genus Trichodesmium contribute significantly to global N₂ fixation (Capone et al., 1997; Karl et al., 2002). The N₂-fixing enzyme nitrogenase is highly sensitive to oxygen, but the genus Trichodesmium is capable of carrying out oxygenic photosynthesis and N₂ fixation in light, although it lacks the protective mechanisms provided by heterocysts. Some protective strategies operative in Trichodesmium spp. have been identified. These include locating the nitrogenase complex exclusively within the diazocytes, forming short stretches of cells (approx. 15%) in the trichomes (Bergman & Carpenter, 1991; Janson et al., 1994; Fredriksson & Bergman, 1995, 1997; Lin et al., 1998), and a lowering of oxygenic photosynthesis around mid-day when N₂ fixation is at its maximum (Berman-Frank et al., 2001).

In the heterocystous cyanobacterium Anabaena PCC 7120, differentiation of a vegetative cell into a heterocyst requires the activation of several genes in a cascade-like mechanism of transcriptional activations (Cai & Wolk, 1997). This differentiation process depends on both the global nitrogen regulator NtcA and the cell differentiation regulatory protein HetR (Wolk, 2000). ntcA encodes a transcriptional regulator that belongs to the CRP family of bacterial regulators (Herrero et al., 2001), which activates the expression of genes involved in nitrogen assimilation upon ammonium withdrawal by binding to specific sites in their promoters. NtcA is required for development and function of mature heterocysts, and for the expression of nitrogen transport and assimilation systems as well as N₂ fixation (Herrero et al., 2001). HetR is a key regulator of heterocyst differentiation (Buikema & Haselkorn, 1991) and is an autoregulated serine-type protease that can degrade itself and possibly other proteins (Zhou et al., 1998). HetR shows enhanced induction within 1–2 h of nitrogen step-down, and within 3.5 h hetR expression is clearly localized to spaced cells, presumably developing pro-heterocysts (Black et al., 1993). Induction of hetR upon nitrogen step-down depends on NtcA (Muro-Pastor et al., 2002). The
presence of hetR in filamentous non-heterocystous strains, including Trichodesmium (Janson et al., 1998; Orcutt et al., 2002; Schiefer et al., 2002), and the requirement of hetR for akinete differentiation (Leganes et al., 1994) suggest that hetR is not exclusively involved in heterocysts differentiation (see Wolk, 2000).

Like heterocysts, diazocytes are the exclusive carriers of nitrogenase and fix nitrogen aerobically in the light, and show morphological and physiological changes. These include a decrease in cyanophycin granules and synthesis of additional membranes (Fredriksen & Bergman, 1997), an increase in glutamine synthetase levels (Carpenter et al., 1992), and an increase in cytochrome oxidase levels (Bergman et al., 1993). Such changes suggest that differential gene expression may be required to generate diazocytes.

As cell differentiation is a fundamental biological process we initiated a study on identification of the molecular basis for the differentiation of the novel N2-fixing cell type (diazocytes) recently discovered in Trichodesmium (Fredriksen & Bergman, 1997; Lin et al., 1998; Berman-Frank et al., 2001). For this purpose, we followed the occurrence and distribution pattern of diazocytes in trichomes and applied the sensitive real-time RT-PCR and SYBR Green I assay to monitor the expression of ntcA, hetR and nifH in cultures of Trichodesmium IMS101 grown under a 24 h light/dark regime, in the absence and presence of ammonia. A comparative search between the Trichodesmium IMS101 and Anabaena PCC 7120 genomes was also performed. Possible mechanisms involved in diazocyte development are discussed.

METHODS

Culture and growth conditions. The Trichodesmium strains examined are listed in Table 1. Cultures of Trichodesmium IMS101 (T. erythraeum) were maintained under a 12 h light/12 h dark regime (L/D cycle) in an amended seawater medium (Prufert-Bebout et al., 1993) at 28 °C. The light intensity was 75 μmol m−2 s−1 and the cultures (600 ml) were aerated at a rate of approx. 200 ml min−1. Under these conditions Trichodesmium IMS101 showed a doubling time of 25–28 h.

Natural populations of Trichodesmium, from the coastal waters of Zanzibar (Indian Ocean at the East Coast of Tanzania), were collected on several occasions between 1995 and 1997 from 125 ml net-towed surface samples (Table 1). Hand-picked colonies, identified as T. erythraeum (Lugomela et al., 2002), were fixed in 1 % borax-buffered formalin and analysed by light microscopy.

\textbf{In situ immunolocalization/light microscopy.} Trichomes were mounted on poly-L-lysine-coated glass slides (Sigma, product no. P0425), fixed and permeabilized in 100 % ethanol overnight at −20 °C (Lin et al., 1998). A rabbit anti-Rhodospirillum rubrum NifH antibody (100-fold dilution) was used as the primary antibody in conjunction with a fluorescein-conjugated secondary anti-rabbit antibody (200-fold dilution) (Molecular Probes, product no. A-11069). Trichomes were examined by light microscopy using an Olympus microscope fitted with U-MWU filters (BP330–385, BA420, DM400).

\textbf{Acetylene reduction assay.} Samples (2 ml) of Trichodesmium cultures were placed in 10 ml serum vials, 10 % (v/v) acetylene was injected and vials were incubated for 1 h under the same growth conditions as those given above. Head-space gas samples were withdrawn, and the ethylene production was measured using a Perkin-Elmer F11 gas chromatograph. Ethylene concentrations were calculated by reference to calibrated ethylene gas standards (Lundgren et al., 2001).

\textbf{Sampling and RNA extraction.} At each sampling time during the 12 h/12 h L/D cycle, cells were collected into sterile plastic tubes and quickly filtered through 5 μm Millipore filters (Whatman, cat. no. 110613). The filters were immediately vortexed in RLT buffer containing β-mercaptoethanol (Qiagen, cat. no. 74104). The filters were discarded and the samples were stored at −80 °C. RNA was extracted using RNeasy Mini Extraction Kit (Qiagen, cat. no. 74104). Relative quantification (see below) of 16S rRNA (GenBank accession no. AF013030) was performed to verify that equal amounts of total mRNA were loaded in the real time RT-PCR runs (results presented in the figure legends).

\textbf{PCR and cloning.} Primer sequences used to amplify part of the sequences of ntcA (AF169961), hetR (AF091323) and nifH (U90952) from Trichodesmium IMS101 were designed accordingly (NCBI database): pntcAP, 5′-TGA TGA TGA AAA CCT TGT CA-3′; pntcAM, 5′-CTC TGC GAT TGC TTG ATG AG-3′; phetRP, 5′-TGA ACC ACA CGC GGG TAA AG-3′; phetRM, 5′-GCT TCA CTA ATG GGC ATC CG-3′; phetR, 5′-CGG TGG CAT TAA GTG TG-3′; phetRM, 5′-ACC TAA ACC GAC ACC AG-3′.

Trichodesmium IMS101 DNA was extracted using the Genomic DNA isolation kit (Qiagen, buffer set cat. no. 19060, QIAprep spin cat. no. 27104) and PCR was carried using HotStar Taq polymerase (Qiagen, cat. no. 203204). PCR products with ntcA, hetR and nifH primers were cloned using the TOPO 2.1 TA cloning kit (Invitrogen, cat. no. 119060) and sequenced by MWG Biotech (Germany) to verify that the primers bound to the right targets. These primers were then used for the SYBR Green I assay below.

\textbf{Real-time RT-PCR and SYBR Green I assay.} The real-time PCR reactions were carried out in an iCycler IQ (Bio-Rad) using a QuantiTect SYBR Green RT-PCR kit (Qiagen, cat. no. 204243). The reaction mixture was prepared to contain 1 × QuantiTect SYBR Green RT-PCR Master mix, 0.5 µl QuantiTect RT Mix, 0.5 µl fluoroscein (1 µM; Bio-Rad, 170-8780), 0.5 µM of each primer, and RNase-free water to 50 µl. The RT-PCR programme was as follows: 50 °C for 30 min, 95 °C for 15 min, 40 cycles at 94 °C for 30 s, 54 °C (55 °C for nifH) for 30 s and 72 °C for 30 s. The fluorescence values were collected at 72 °C. The relative cDNA quantities of ntcA, hetR and nifH, and hence mRNA, were determined using serial dilutions of Trichodesmium IMS101 DNA (330 ng ml−1) as a standard (at least four dilutions were included in each measurement). Following each run, a melt curve analysis step was performed according to the

\begin{table}
\centering
\begin{tabular}{|l|l|}
\hline
\textbf{Strain/population} & \textbf{Origin/sampling site} \\
\hline
T. erythraeum IMS101 & Cultured isolate, non-axenic \\
T. erythraeum NC2* & Cultured isolate, axenic \\
T. erythraeum TR-1* & Cultured isolate, axenic \\
T. erythraeum XIII* & Cultured isolate, axenic \\
T. erythraeum† & Natural population, Indian Ocean \\
\hline
\end{tabular}
\end{table}

*Isolated by Dr. J. Waterbury (WHOI, Woods Hole, USA); NC2 identical to T. IMS101.
†Collected 2 May 1995 (130 trichomes); 20 May 1995 (100); 20 March 1996 (300); 10 April 1996 (102); 15 January 1997 (500).
iCycler iQ manual to verify that the PCR products from the mRNA and DNA were the same, and that primer dimers were absent or if present that they were not stable at 72°C and hence not incorporated into the datasets. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

**Genome analysis.** A comparative genomic analysis between the heterocystous *Anabaena* PCC 7120 (http://www.kazusa.or.jp/cyano/Anabaena/index.html) and *T. erythraeum* IMS101 (http://genome.jgi-psf.org/draft_microbes/trier/trier.home.html) was undertaken using BLAST.

**Effects of PatS.** The synthetic PatS-5 peptide (a generous gift from Dr J. W. Golden, Texas A & M, Texas, USA), known to prevent heterocyst development in *Anabaena* PCC 7120 (Yoon & Golden, 1998), was added to *Trichodesmium* IMS101 cultures at concentrations ranging from 1 to 10 μM. After 36 h, the occurrence of diazocytes was analysed using *in situ* immunolabelling of NifH and the trichomes were examined by light microscopy (as above).

**RESULTS AND DISCUSSION**

It has been proposed that the occasionally observed perceptibly lighter regions in *Trichodesmium* spp. may be sites for N₂ fixation (Carpenter & Price, 1976; Bryceson & Fay, 1981). Light microscopy analysis of cultured *Trichodesmium* IMS101 (*T. erythraeum*; Janson et al., 1999) and trichomes from five natural populations of *T. erythraeum* (Table 1), collected in the Indian Ocean, revealed non- or less-granulated regions of cells within the trichomes with different optical properties making them appear as ‘lighter’ (Fig. 1). These regions were flanked by cells of a darker more granulated appearance. By using *in situ* immunolocalization/light microscopy of dinitrogenase reductase (NifH) we were able to demonstrate that these non-granulated or lighter regions corresponded to the regions of NifH-containing diazocytes (Fig. 2). Such diazocyte regions were present in all *Trichodesmium* strains examined here, whether axenic or not (Tables 1 and 2).

This finding allowed a light microscopy search for diazocyte regions in a large number of trichomes in five naturally growing *T. erythraeum* populations (Tables 1 and 2), the purpose being to document their occurrence and distribution along trichomes. The regions were intercalary, terminal, or occasionally at both locations in the trichomes.

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**Fig. 1.** Light micrographs of individual trichomes from naturally grown *T. erythraeum* colonies showing the occurrence of the lighter, non-granulated cell regions at varying positions along the trichomes: (A) intercalary, central; (B) terminal; (C) intercalary, close to end of trichome; and (D) terminal and intercalary. Bars, 80 μm.

**Fig. 2.** Light micrographs depicting the coincident location of the non-granulated cells and the NifH-labelled cells, representing diazocytes, in one individual trichome of *Trichodesmium* IMS 101. (A) An intercalary region composed of a stretch of non-granulated cells (bracketed); (B) the same trichome labelled with an anti-NifH antibody and viewed using fluorescence microscopy. The presence of nitrogenase (diazocytes) is seen as blue fluorescence. Bar, 8 μm.
The intercalary diazocyte regions were mostly positioned in central areas of the trichomes (Fig. 1A, D), and most trichomes had one or two regions (Fig. 3A). In cultured Trichodesmium IMS101 about 60% of the trichomes had only one diazocyte region. Higher frequencies of regions (up to four regions) were present in the natural populations of T. erythraeum, often composed of longer trichomes (Fig. 3B). The latter is possibly due to the more favourable growth conditions in the field. Trichomes composed of fewer than about 40 cells seemed to lack diazocyte regions (Fig. 3B). Presumably, such non-diazocytous short trichomes are formed by fragmentation. The most fragile junction of Trichodesmium trichomes is that between a diazocyte and the nearby vegetative cell (authors’ observations).

The proportion of differentiated, lighter (non-granulated) cells per trichome was strikingly similar (approx. 17%) in both the cultured and the naturally grown populations (Table 2). This percentage also coincided with the frequency in diazocyte regions identified (approx. 14%), irrespective of site of collection or Trichodesmium axenicity (Table 2). As the growth conditions for the populations examined here were radically different, we hypothesized that a developmental rather than an environmental mechanism underpins diazocyte formation in Trichodesmium. In heterocystous cyanobacteria such as Nostoc and Anabaena the percentage of heterocysts is also constant, peaking at 5–10%, and hetR is a master gene for the heterocyst differentiation (Buikema & Haselkorn, 1991; Adams & Duggan, 1999). The role of hetR in some non-heterocystous

### Table 2. Percentage of differentiated cells (non-granulated) and cells with NifH (diazocytes) in cultured and naturally grown Trichodesmium strains

Mean percentages are given and figures in parentheses denote number of cells counted.

<table>
<thead>
<tr>
<th></th>
<th>Cultivated isolates</th>
<th>Natural populations</th>
<th>References</th>
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<tbody>
<tr>
<td>Cell differentiation (non-granulated cells)</td>
<td>18% (150)*</td>
<td>16% (1130)†</td>
<td>This study</td>
</tr>
<tr>
<td>Diazocytes (NifH-labelled cells)</td>
<td>14% (700)*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>15% (300)‡</td>
<td>–</td>
<td>Lin et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>14% (1500)§‖</td>
<td>Fredriksson &amp; Bergman (1995)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10% (25)¶§‖</td>
<td>Janson et al. (1994)</td>
<td></td>
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</tbody>
</table>

*The three axenic Trichodesmium strains (Table 1).  
†T. erythraeum, Indian Ocean.  
‡The non-axenic Trichodesmium IMS101.  
§T. tenue, T. thiebautii, T. erythraeum, the Caribbean Sea.  
‖The diazocyte frequency ranged from 5 to 24% depending on the time of day.  
¶T. contortum, Caribbean Sea.

(Fig. 1A–D). The intercalary diazocyte regions were mostly positioned in central areas of the trichomes (Fig. 1A, D), and most trichomes had one or two regions (Fig. 3A). In cultured Trichodesmium IMS101 about 60% of the trichomes had only one diazocyte region. Higher frequencies of regions (up to four regions) were present in the natural populations of T. erythraeum, often composed of longer trichomes (Fig. 3B). The latter is possibly due to the more favourable growth conditions in the field. Trichomes composed of fewer than about 40 cells seemed to lack diazocyte regions (Fig. 3B). Presumably, such non-diazocytous short trichomes are formed by fragmentation. The most fragile junction of Trichodesmium trichomes is that between a diazocyte and the nearby vegetative cell (authors’ observations).

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cyanobacteria, including *Trichodesmium* (Janson et al., 1998, Orcutt et al., 2002; Schiefer et al., 2002), is still obscure.

In order to search for the role of *hetR* and its possible connection to diazocyte formation in *Trichodesmium* we made use of the real-time RT-PCR methodology and the SYBR Green I assay. This is a powerful technique for detecting and quantifying low mRNA expression levels (Gibson et al., 1996; Heid et al., 1996; Lie & Petropoulos, 1998). We first examined the expression of *hetR* and the global regulatory gene involved in nitrogen control and heterocyst differentiation, *ntcA* (Herrero et al., 2001), in N$_2$-fixing cultures. As nitrogenase is confined to diazocytes, the expression of *nifH*, the gene encoding dinitrogenase reductase, was simultaneously followed. The specificity of the three primer pairs used was checked by amplification and cloning of the fragments generated, followed by sequencing and comparisons with already published cyanobacterial sequences using BLAST provided at the NCBI database site (http://www.ncbi.nlm.nih.gov/BLAST/). The characteristic $T_m$ of the amplified fragments were easily distinguished from the $T_m$ of non-specific products and primer dimers on a melt curve chart (data not shown). Measurements of fluorescence intensity were only performed at temperatures above the $T_m$ of the primer dimers and below that of the specific PCR products. Product identification was performed by comparing the respective $T_m$ on the melt curve charts and the respective molecular size on agarose gels.

The expression of *ntcA*, *hetR* and *nifH* in samples of *Trichodesmium* IMS101 was followed over 12 h/12 h L/D periods. As seen in Fig. 4, *ntcA* was constitutively expressed at moderate levels through the diurnal cycle. In contrast, *hetR* expression showed a distinct diurnal rhythmicity. It increased sharply from 20:00 h and reached a fourfold higher expression 2 h into the dark phase. Expression then decreased to a minimum just before 08:00 h in the morning, when the light was again switched on. The data also show that *hetR* is still expressed, albeit at low levels, under the more nitrogen-replete periods (daytime). The timing in the *hetR* expression pattern was persistent in all experiments ($n=10$), although expression levels at the peak varied between 3- and 10-fold.

As expected, the peak in *nifH* expression occurred at midday (Fig. 4), about 2 h before the maximum nitrogenase activity was reached (measured as acetylene reduction). These findings corroborate those reported previously for *nifH* expression in *Trichodesmium* IMS101 using Northern blots (Chen et al., 1998; Dominic et al., 1998), immunolocalization of NifH (Fredriksson & Bergman, 1995) and acetylene reduction assays (Mulholland & Capone 1999).

While NifH synthesis was below the detection level of Northern blots when the light was turned off (20:00), we were able to demonstrate by using the more sensitive real-time RT-PCR technique that initiation of *nifH* transcription commenced at about midnight and slowly increased until the light was again turned on at 08:00; it then increased rapidly (Fig. 4). This finding may explain the presence of the nitrogenase protein during the night (Zehr et al., 1993; Fredriksson & Bergman, 1995; Chen et al., 1998), and further confirms that the nitrogenase protein abundance is reflected in the *nifH* transcription pattern (Chen et al., 1998).

To investigate the possible role of *ntcA* and *hetR* in relation to N$_2$ fixation and diazocyte formation a source of combined nitrogen was added to the N$_2$-fixing *Trichodesmium* IMS101 cultures (Fig. 5). When 100 $\mu$M NH$_4$Cl was added at 20:00 h ($n=4$), *ntcA* expression decreased and the upshift in *hetR* expression, seen under N$_2$-fixing conditions at 22:00 (Fig. 4), was diminished and followed by a slow continuous decrease. Likewise, *nifH* expression was negatively affected (Fig. 5), and no acetylene reduction activity was recorded. The upshift seen at the end of the light period (Fig. 5) was probably due to exhaustion of NH$_4^+$ from the growth medium. Higher concentrations of NH$_4^+$ were avoided as they may negatively affect *Trichodesmium* growth. A repression of *ntcA*, *hetR* and *nifH* expression and heterocyst differentiation by combined nitrogen is well known for heterocystous cyanobacteria (Herrero et al., 2001; Adams & Duggan, 1999), and the expression of *ntcA* and *hetR* are mutually dependent on each other (Muro-Pastor et al., 2002).

To get further insights into the possible genetic background to diazocyte development, a comparative genomic analysis between the heterocystous *Anabaena* PCC 7120 and *T. erythraeum* IMS101 was undertaken using BLAST. Genes involved in regulating early stages of heterocyst differentiation in *Anabaena* PCC 7120 (Wolk, 2000) were identified in *Trichodesmium* IMS101 while several others were not (Table 3). As expected, *ntcA* and *hetR* were

![Fig. 4. Diurnal expression profiles of ntcA (□), hetR (○) and nifH (△) in Trichodesmium IMS101. Expression was assayed using real-time RT-PCR, during a 12/12 h L/D cycle. SQ, starting quantities. SQ 16 rRNA = 5.14 (SD ± 0.42, n = 9). The black bar represents the 12 h dark period and the white bar the 12 h light period.](http://mic.sgmjournals.org)
identified and showed a high amino acid identity. Other genes identified were *hanA*, encoding a DNA-binding protein necessary for heterocyst initiation (Nagaraja & Haselkorn, 1994; Khudyakov & Wolk, 1996), and *devR*, a response regulator also essential for heterocyst formation (Campbell *et al*., 1996). Among the genes known to control heterocyst pattern formation (individual heterocyst distribution at regular intervals), *patB* (a putative transcription regulator; Liang *et al*., 1993) was found but not *patA* or *patS* (Liang *et al*., 1992; Yoon & Golden, 1998, 2001). In heterocystous cyanobacteria, differentiation may be initiated in a cluster of cells (proheterocysts), but only one develops into a mature heterocyst, while the others regress to become vegetative cells (Wilcox *et al*., 1973). PatS is presumed to regulate this resolution into individual heterocysts via lateral inhibition (Yoon & Golden, 1998, 2001). Diazocytes in contrast always remain in groups of about 3–20 cells (Figs 1 and 2; Lin *et al*., 1998). Additions of the inhibitory synthetic PatS-5 peptide to *Trichodesmium IMS101*, at concentrations known to prevent heterocyst development (Yoon & Golden, 1998, 2001), had no influence on the frequency or the distribution of the NifH-labelled diazocytes. This also supports the absence of *patS* in *Trichodesmium IMS101*. Not unexpectedly, genes involved in the synthesis of the thick heterocystous cell envelope, composed of polysaccharides and glycolipids (Wolk, 2000), were not identified in the genome of *Trichodesmium* (Table 3). In spite of fixing N$_2$ aerobically during the day, diazocytes lack the equivalent external cell-wall layers (Bergman & Carpenter, 1991; Fredriksson & Bergman, 1997).

![Fig. 5. Effects of adding combined nitrogen (NH$_4^+$) on diurnal expression profiles in *Trichodesmium* IMS101.](image)

Expression was assayed, as in Fig. 4, for *ntcA* ( ), *hetR* ( ) and *nifH* ( ) after the addition of 100 µM NH$_4$Cl at 20:00 h (*n* = 4). Note the difference in scale on the *y*-axes compared to Fig. 4. Upshifts in expression levels at the end of the light phase are due to exhaustion of (NH$_4^+$). SQ, starting quantities. SQ 16 rRNA = 2-17 (SD ± 0.45, *n* = 9). The black bar represents the 12 h dark period and the white bar the 12 h light period.

Table 3. Gene homologies between *Trichodesmium IMS101* and *Anabaena* sp. strain PCC 7120

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amino acid identity (%)</th>
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<tr>
<td><em>ntcA</em></td>
<td>78</td>
</tr>
<tr>
<td><em>hetR</em></td>
<td>76</td>
</tr>
<tr>
<td><em>hanA</em></td>
<td>75</td>
</tr>
<tr>
<td><em>devR</em></td>
<td>75</td>
</tr>
<tr>
<td><em>devH</em></td>
<td>69</td>
</tr>
<tr>
<td><em>patB</em></td>
<td>58</td>
</tr>
<tr>
<td><em>hetP</em>, <em>hetC</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>patA</em>, <em>patS</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>devA</em>, <em>devB</em>, <em>devC</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>hepK</em>, <em>hepA</em>, <em>hepB</em>, <em>hepC</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>hglC</em>, <em>hglD</em>, <em>hglE</em>, <em>hglK</em></td>
<td>ND</td>
</tr>
</tbody>
</table>

Ever since the discovery in 1991 (Bergman & Carpenter, 1991) of the nitrogenase-containing cells (the diazocytes: Fredriksson & Bergman, 1997), no studies have been directed towards understanding the mechanisms related to diazocyte development and pattern formation in *Trichodesmium*. The data presented here, however, clarify some novel aspects, and probably extend to other cyanobacteria such as *Katagnymene* spp. which show a similar N$_2$-fixing behaviour (Lundgren *et al*., 2001). First, we demonstrate that the morphologically distinct lighter stretches seen in certain *Trichodesmium* trichomes are the nitrogenase-containing diazocyte zones. Secondly, that individual trichomes may have multiple, regularly spaced diazocyte regions, the number and location of which is related to growth conditions and length of the trichomes. Thirdly, that a developmentally regulated diazocyte differentiation exists, as there is a constant mean frequency (approx. 15%) of diazocytes under the highly varying external conditions. This in turn led us to argue that *hetR* may be an actor in diazocyte development. For instance, we were able to show a distinct diurnal rhythmicity in *hetR* expression in a cyanobacterium, while *ntcA* was more stably expressed during the 24 h growth cycle. The peak in *hetR* expression preceded the onset of *nifH* expression by the same time span (6–8 h) as that noted between *hetR* and *nifH* gene expression in heterocystous cyanobacteria, when grown at similar doubleting times (for *Trichodesmium* 25–28 h under our conditions) (Fig. 4; Adams & Duggan, 1999; Yoon & Golden, 2001). In order to maintain a highly constant mean daytime frequency of diazocytes (present study; Fredriksson & Bergman, 1995; Lin *et al*., 1998) new diazocytes must regularly develop as trichomes grow.
Moreover, there is a diurnal rhythmicity in the frequency of cells with NiF (diazocytes) in natural *Trichodesmium* populations, with maximum frequencies being reached in the daytime (approx. 24 %) and a minimum (approx. 5 %) just before dawn (Fredriksson & Bergman, 1995). This suggests a diurnal redifferentiation of diazocytes (diazocytes are, unlike heterocysts, not permanently differentiated but able to divide; Fredriksson & Bergman, 1997), and that the time span for differentiation of new diazocytes is located between the peak in *hetR* and the onset of *nifH* expression (Fig. 4). *hetR* expression is considerably reduced (as is that of *ncaA* and *nifH*) in the presence of an external nitrogen source (Fig. 5), which is also typical for all other *hetR*-dependent heterocystous cyanobacteria (Adams & Duggan, 1999). The presence of some crucial early heterocyst differentiation genes in the genome of *Trichodesmium* also points to some shared cell differentiation mechanisms. However, to fully verify the linkage between *hetR* expression and diazocyte formation the construction of *Trichodesmium hetR* mutants is required, and such attempts are now under way.

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